Antitumor Effect of Combination of Hyperthermotherapy and 5-Aminolevulinic acid (ALA)

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Abstract. *Background: 5-Aminolevulinic acid (ALA) is a precursor of heme. ALA is used as a photosensitive substance in photodynamic diagnosis (PDD) and photodynamic therapy (PDT) because heme metabolism is abnormal in tumor cells and a photosensitive metabolite of heme synthesis from ALA, protoporphyrin IX (PpIX), specifically accumulates in tumors. We investigated the enhancement of the antitumor effect by combination of ALA and hyperthermotherapy (HT) using a transplanted tumor model with Lewis lung carcinoma cells (3LL) in mice. Materials and Methods: Animals were divided into four test groups: control (untreated), HT, and HT plus ALA (100 or 300 mg/kg) groups, and HT by bathing at 43˚C for 20 min was performed at five days after transplantation. ALA was administered once at the above doses three hours before HT by intraperitoneal injection. Results: The tumor sizes at five days after HT were 5.2- and 2.6-times greater than those at the time of HT in the control and HT groups, respectively. In contrast, PpIX accumulation in the tumor region was noted three hours after ALA administration, the HT+ALA group given at 100 or 300 mg/kg of ALA inhibited tumor growth to 1.3- and 1.1-times increases in the tumor size. Conclusion: Therefore, ALA administration markedly enhanced the tumor growth-inhibitory effect of HT.*

Hyperthermotherapy (HT) utilizes the phenomenon that cancer cells are inactivated by heating tumorous regions, and it is widely recognized as being a cancer therapy of low-invasiveness causing less adverse effects. It has been reported that heating of cancer cells at 42˚C or higher temperature induces necrosis and apoptosis (1, 2), and instability of the cell membrane structure, protein degeneration, and inactivation of the DNA replication system (3-6). HT is not administered alone because its antitumor effect is mild, and a synergistic effect with chemotherapy and radiotherapy is expected. van der Zee *et al*. reported on therapeutic outcomes of advanced uterine cervical cancers (stage 2b, 3b, and 4) treated with HT-combined radiotherapy in 2000, in which the complete response rate was 57% in the group treated with radiation alone, but it markedly increased to 83% in the HT-combined group, and the 3-year survival rate also markedly increased in the HT-combined group (51%) compared to that (27%) of the group treated with radiation alone (7). This report attracted attention to HT for combination therapy of cancer. For HT to play an important role in multidisciplinary treatment of malignant tumors, it may be necessary to improve the effect of HT alone, and the development of a sensitizer to enhance the effect of HT is expected. Several substances have been reported to exhibit an HT-sensitizing effect, but clinical application has not been reached because of problems caused by adverse effects.

5-Aminolevulinic acid (ALA) is a natural delta amino acid which is not a component of proteins. ALA is widely distributed in both plant and animal cells, and is a common precursor of tetrapyrrole compounds including porphyrin, chlorophylls and heme (8). It is usually synthesized by condensation from glycine and succinyl CoA by ALA synthase (EC 2.3.1.37) in animal mitochondria, then transported across both mitochondrial inner and outer membranes into the cytosol and converted to co-proporphyrinogen III through four steps by a metabolic enzyme in the cytosol (9, 10). Co-proporphyrinogen III returns to the mitochondria by ATP-binding cassette sub-family B member-6 (ABCB6) transporter and is metabolized

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to protoporphyrin IX (PpIX) through two further steps. Ferrochelatase (EC 4.99.1.1) catalyzes the last step in heme biosynthesis by inserting ferrous iron into PPIX to produce heme in the mitochondria (11).

In humans, it is well-known that the metabolic pathway of ALA includes the heme biosynthesis pathway and biokinetics in extracellular treatment of ALA. When ALA is administered orally, it is absorbed rapidly *via* human proton-coupled amino acid transporter-1 (hPAT1) in the upper part of the gastrointestinal tract (12), transported into cells by peptide transporter-1 (PEPT1) transporter and metabolized to heme using the same metabolic pathway of endogenous ALA in normal cells. Heme produced from the administered ALA is utilized as hemoglobin and hemoproteins. Heme is closely involved in mitochondrial energy metabolism as a component of the electron transport chain systems, complexes II, III, and IV and cytochrome c.

On the other hand, the administration of ALA rapidly and preferentially increases the level of intracellular PpIX, a photosensitizing porphyrin in cancer cells. Together with light irradiation, ALA-induced intracellular accumulation of PpIX is also used for photodynamic diagnosis (PDD) and photodynamic therapy (PDT) to identify and kill tumor cells, resulting in a new strategy for cancer diagnosis and therapy (8) in neurosurgery (13), urology (14), otorhinolaryngology (15) and gastroenterology (16, 17).

In this study, using a transplanted tumor model with Lewis lung carcinoma cells (3LL) in mice, we investigated the enhancement of the antitumor effect by combination of ALA and HT.

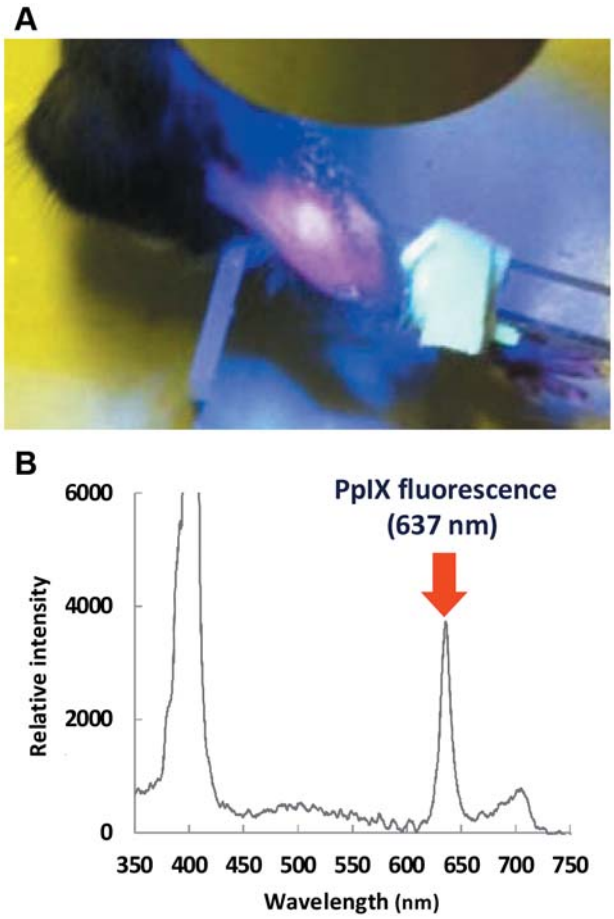

Figure 1. *Confirmation of red fluorescence of protoporphyrin IX (PpIX) accumulated in the subcutaneous tumor region in the thigh. The right thigh tumor was exposed three hours after 100 mg/kg 5-aminolevulinic acid (ALA) administration and irradiated with blue light, and intense red fluorescence was confirmed using a 405-nm simple light source (A). Fluorescence spectrum of the subcutaneous tumor region in the thigh. A fluorescence spectrum of the subcutaneous tumor region in the thigh shown in Figure 1A was obtained. A PpIX-specific peak was present near 637 nm (arrow). The peak near 405 nm was that of the excitation light (B).*

Materials and Methods

Animals and tumors. Female C57BL/6J mice were purchased from Japan SLC, Inc. (Shizuoka, Japan). Animals were give free access to food, MF (Oriental Yeast Co., Ltd., Tokyo, Japan) and tap water, and maintained in a controlled room at 24-26˚C room temperature and 60±5% humidity under a 12-hour fluorescent lighting cycle.

For tumor cells, 3LL line which originated from the C57 strain was used (18, 19). For *in vitro* maintenance, cells were cultured in Dulbecco's modified Eagle's medium (D-MEM) (high glucose) with L-glutamine and phenol red (Wako Pure Chemical Industries, Ltd., Osaka, Japam) with 10% fetal bovine serum (Hyclone Laboratories Inc, Utah, USA). 3LL cells (5×10^5/0.05 ml) were subcutaneously implanted into the right thigh of 7-week-old mice. At 7 or 8 days after implantation when tumors had reached 2-3 mm, the animals were divided into four groups consisting of six animals each, with tumor sizes similar among the groups. The animals were then maintained until tumors grew to 4-5 mm. This experiment was performed after obtaining approval by the Animal Experiment Committee of Louis Pasteur Center for Medical Research (No. 2012-2).

Test groups. The animals were assigned to one of four groups: control (untreated), HT treatment alone (HT), HT puls low-dose ALA treatment (HT-ALAL), and HT plus high-dose ALA treatment (HT-ALAH), respectively. The HT-ALAL and HT-ALAH groups received *i.p.* administration of 0.3 ml of ALA solution (5-Aminolevulinic acid hydrochlolide, SBI Pharmaceuticals Co., Ltd., Tokyo, Japan) prepared with pure water and adjusted to 100 and 300 mg/kg, respectively.

Confirmation of porphyrin accumulation in tumor. Tumor-bearing mice were similarly prepared separately from the above test groups to confirm porphyrin accumulation in the tumors. Porphyrin accumulation in the subcutaneous tumors was confirmed three hours after *i.p.* administration of 100 mg/kg ALA solution: tumors were irradiated with 405 nm blue visible light; the spectrum of emitted fluorescence was acquired using a spectrometer-equipped purple semiconductor laser system for fluorescence diagnosis research, VLD-M1 (SBI Pharmaceuticals Co., Ltd., Tokyo, Japan) and the peak of porphyrin red fluorescence near 635 nm was detected.

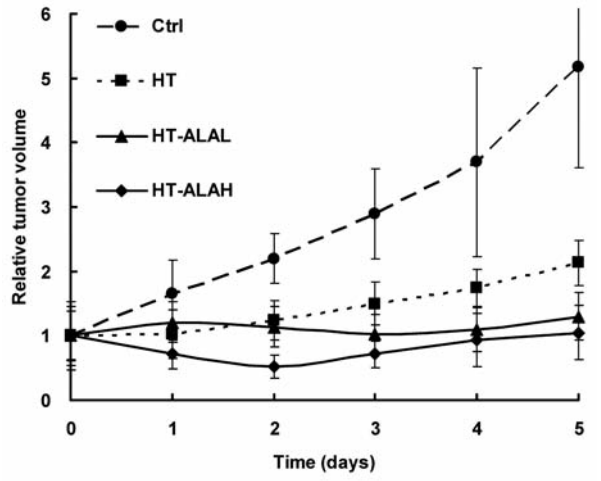

Figure 2. *Growth curve of subcutaneous tumor in the thigh in each treatment group. Ctrl: control (untreated) group; HT: hyperthermotherapy alone; HT-ALAL: HT with low-dose 5-aminolevulinic acid (ALA) and HT-ALAH: HT with high-dose ALA. HT and ALA were administered on day 0. The plots represent the mean±standard deviation (n=6) of the relative tumor volume. *p<0.05, **p<0.01: vs. HT by the Tukey-Kramer method.*

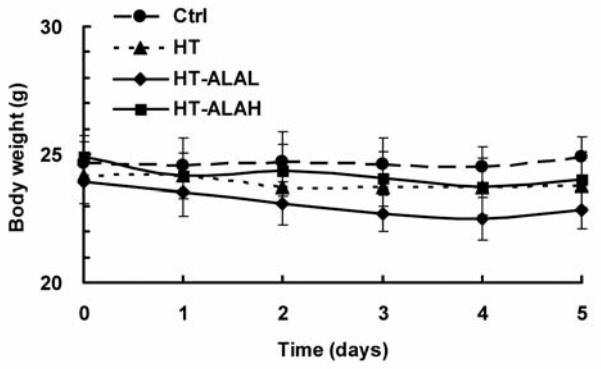

Figure 3. *Time-course body weight changes in each treatment group. Ctrl: control (untreated) group; HT: hyperthermotherapy alone; HT-ALAL: HT with low-dose 5-aminolevulinic acid (ALA); HT-ALAH: HT with high-dose ALA group. The plots represent the mean±standard deviation (n=6) of the body weight.*

Hyperthermotherapy. Three hours before HT, the HT-ALAL and HT-ALAH groups received *i.p.* administration of 100 and 300 mg/kg of ALA solutions, respectively. HT was performed using a water bath. Only the right thighs of the tumor-bearing mice were placed in a 43˚C water bath for 20 minutes.

Measurement of tumor volume. The major (a) and short (b) axes of tumors were measured daily using calipers, and the tumor volume (V) was calculated by the following equation: $V=3/4\pi\times a/2\times(b/2)^2$.

Pathological analysis. Mice were selected from each group at day 10 after HT, sacrificed, autopsied, and the subcutaneous tumor and lung were excised. Tissue sections were prepared from the excised specimens.

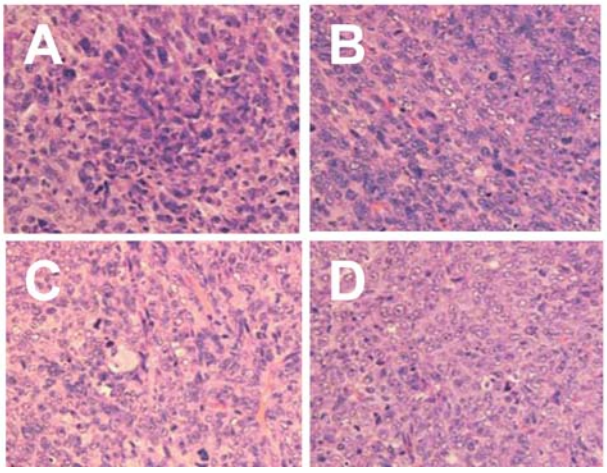

Figure 4. *Hematoxylin-eosin-stained subcutaneous tumor tissues at day 10 in each group. In the control (untreated) group (A), necrosis was noted in the center. The nuclei were sufficiently stained in the surrounding cancer cells, showing active cell division. In the group treated with hyperthermotherapy alone (B), vacuolated cells and cells with nuclear fragmentation were occasionally noted around active tumor cells containing sufficiently stained nuclei. In the group treated with hyperthermotherapy with low-dose 5-aminolevulinic acid (ALA) (C), cells containing sufficiently stained nuclei showing cell division activity were occasionally noted, and vacuolated cells and cells with nuclear fragmentation were present around them. In the group treated with hyperthermotherapy with high-dose ALA (D), the number of stained nuclei was lower, and vacuolated cells and cells with nuclear fragmentation were widely distributed, showing that only a few cells had proliferative ability. Original magnification, ×400.*

Statistical analysis. The Student's *t*-test was used to compare the control and HT groups, followed by multiple comparison of three groups: HT, HT-ALAL, and HT-ALAH groups using the Tukey-Kramer method. $p<0.05$ was considered significant.

Results

Confirmation of PpIX accumulation in the transplanted tumor region in tumor-bearing mice. Tumor-bearing mice were killed by cervical dislocation three hours after ALA administration. When the right thigh tumor was exposed and irradiated with blue light using a 405-nm simple light source, intense red fluorescence was noted in the tumor region (Figure 1A). When the fluorescence spectrum in this region was obtained, a PpIX-specific peak was present near 635 nm (Figure 1B).

Enhancement of the antitumor effect of HT by ALA. The time-course of the relative tumor volume in each group is shown in Figure 2. The tumor volume increased with time in the control group, but the slope of the growth curve became moderate in the group treated with HT alone, showing an

inhibition of volume increase. In the HT-ALAL and HT-ALAH groups, the increase in tumor volume was more strongly inhibited than that in the HT group. In particular, the tumor volume decreased up to two days after HT in the HT-ALAH group. The relative tumor volume at two days after HT was 1.2±0.3 (mean±standard deviation) in the HT group, showing significant tumor growth inhibition compared to that (2.2±0.4) in the control group ($p<0.01$). The tumor volume in the HT-ALAL group was 1.1±0.3, showing no significant difference from that in the HT group, but was 0.5±0.2 in the HT-ALAH group, showing significant tumor growth inhibition ($p<0.01$).

At five days after HT, the tumor volume was 2.1±0.3 in the HT group, showing a significant tumor growth inhibition compared to that (5.2±1.6) in the control group ($p<0.01$). In addition, significant tumor growth inhibition compared to that in the HT group were noted in both HT-ALAL (1.3±0.4) and HT-ALAH (1.1±0.4) groups ($p<0.01$, respectively). Regarding changes in the body weight, no ALA concentration-dependent weight loss was noted in any group throughout the 5-day period (Figure 3).

Inhibition of distant metastasis by combination of ALA and HT. The mice of each group were euthanized at 10 days after HT. The subcutaneous tumor implanted in the right thigh and lung tissue were excised and subjected to H&E staining and observation. In subcutaneous tumor tissue (Figure 4), nuclear fragmentation and vacuolation of cells were observed in the HT group, compared to those in the control group, and these changes were marked in the HT-ALAL and HT-ALAH groups.

Discussion

It was demonstrated here that ALA enhances the antitumor effect of HT. A significant tumor growth-inhibitory effect was obtained in the group treated with HT alone, but tumor growth was not inhibited throughout the study period. In contrast, tumor growth was strongly inhibited throughout the study period in the HT-ALAL group, and the relative tumor volume remained 1.3 on day 5 after HT. A point worthy of special mention is the antitumor effect observed in the HT-ALAH group: the tumor volume decreased until two days after HT. The tumor slowly grew thereafter, but the final relative tumor volume at five days after HT was 1.1, showing a promising antitumor effect.

It is well-known that ALA induces tumor-specific PpIX accumulation, regardless of the route of administration. Ishizuka *et al*. reported that when mouse liver cancer cells (MH134) were cultured in the presence of 1 mM ALA, the cellular PpIX levels increased, and when ALA (24 mM, 0.25 ml) was intravenously injected into normal and MH134 tumor-bearing mice, the plasma and urinary porphyrin levels markedly increased in tumor-bearing mice (20). The enhancement of the antitumor effect of HT by concomitant ALA may have been closely associated with an ALA metabolite, PpIX, specifically accumulated in the subcutaneous tumor. In ALA-PDT, singlet oxygen produced by exciting PpIX, specifically accumulated in tumors with light, exhibits an antitumor effect on cancer cells. Yamamoto *et al*. cultured rat glioma cells with ALA, and excited PpIX accumulated in the cells by radiation, which induced production of reactive oxygen species (ROS) in the cells and led them to death (21). An antitumor effect of combination of ultrasound and photosensitizers, including 5-ALA, has recently been reported, suggesting that the combination effect of singlet oxygen production induced by photosensitizer excitation with sonoluminescent light and ultrasound-induced temperature elevation in tumor regions is the mechanism of the antitumor effect (22, 23). Ohmura *et al*. reported that combination of ALA and weak-focused ultrasound (10 W/cm^2, 1.04 MHz, 5 min) shrank a glioma implanted in rat brain without elevating the temperature of the tumor region (24). Elucidation of the mechanism of this antitumor effect is awaited. Regarding the combination of HT and ALA employed by us, Chibazakura *et al*. reported that ALA promoted heat stress-induced tumor cell death without light irradiation, the cell death rate was correlated with the PpIX levels accumulated in the tumor, and ROS were produced in the cells under this condition (25), which is supported by our results. However, the mechanism of the antitumor effects exhibited through combination of heat, having a relatively low energy and ALA, may be different from the mechanism of ROS production induced by exciting PpIX specifically accumulated in cancer cells by light irradiation, similar to the mechanism of established ALA-PDT. Further investigation of the mechanism of the antitumor effect obtained in this study is necessary.

Further study should determine whether the combination of ALA and HT might also inhibit lung metastasis of the subcutaneous tumor of the thigh. Tumor surveillance by the immune system is closely involved in cancer growth and metastasis. Regarding metastasis, the role of natural killer (NK) cells has been shown to be particularly important (26). Regarding lung metastasis of 3LL cells used in this study, it has been reported that NK cells work as effector cells (27). Skivka *et al*. applied ALA-PDT to 3LL cells, and observed an increase in tumor-infiltrating mononuclear cells and activation of intraperitoneal macrophages (28). It has also been recently considered that crosstalk between NK and dendritic cells (DC) is responsible for the coordination of innate immunity by NK cells and adaptive immunity and works toward induction of specific immunity including cytotoxic T-cells (29). Through the combination of ALA and HT, cancer antigens presented on heat-shock

protein (HSP) molecules may be utilized for antigen presentation by DCs (8). From our preliminary experiment, the combination of ALA and HT not only exhibited an antitumor effect on the primary lesion but also inhibited metastasis (data not shown), and this is very important with regard to medical application, for which further precise study including detailed analysis of the mechanism should be essential to conclude.

ALA is a natural amino acid widely present in nature. It is marketed as a drug for PDD and PDT, and its high-level safety is established on abundant non-clinical and clinical studies. Since HT alone exhibits only a relatively mild antitumor effect, it is administered in combination with radiotherapy, chemotherapy, and immunotherapy in most cases. Thus, sensitizers of HT have been widely sought. Many studies on the combination of HT with anticancer drugs, such as alkylating agents and metal complexes, have been reported, and the radical production-inducing effect common to these substances is considered to be the sensitization mechanism of HT. However, most substances with an HT-sensitizing action discovered so far induce many adverse effects and have not reached clinical application. When the ALA dose used in our experiment was converted to that in humans, it was similar to the dose used in PDD and PDT in which safety has been established, and the PpIX level in the body after oral ingestion of ALA at this dose returns to the baseline level within 24 hours after ingestion. No ALA-induced body weight change was noted in our experiment, and no adverse effects occurred (Figure 3). PpIX derived from administered ALA accumulates in tumor cells, but it is rapidly metabolized to heme in normal cells. Thus, the risk of normal cell damage is very low when ALA is administered in combination with HT. HT was performed only once, but, considering its low-invasiveness and high-level safety of ALA, several administrations of ALA-hyperthermia are possible, for which a higher antitumor effect is expected.

In summary, it was clarified that the combination with ALA enhanced the antitumor effect of HT in the 3LL transplanted mouse tumor model. The combination of HT with ALA not only enhanced the antitumor effect on the implanted tumor but also had no adverse effects. Although detailed analysis of the action mechanism is essential, the combination with ALA adds a new value to HT, which is of low invasiveness but exhibits only a mild effect. This treatment method may be used as a novel cancer therapy, and may increase the importance of the role of HT in multidisciplinary treatment.

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References

1 Bergs JW, Haveman J, Ten Cate R, Medema JP, Franken NA and Van Bree C: Effect of 41˚C and 43˚C on cisplatin radiosensitization in two human carcinoma cell lines with different sensitivities for cisplatin. Oncol Rep *18*: 219-226, 2007.
2 Hildebrandt B and Wust P: Interactions between hyperthermia and cytotoxic drugs. Cancer Treat Res *134*: 185-193, 2007.
3 Franke K, Kettering M, Lange K, Kaiser WA and Hilger I: The exposure of cancer cells to hyperthermia, iron oxide nanoparticles, and mitomycin C influences membrane multidrug resistance protein expression levels. Int J Nanomedi *8*: 351-363, 2013.
4 Hildebrandt B, Wust P, Ahlers O, Dieing A, Sreenivasa G, Kerner T, Felix R and Riess H: The cellular and molecular basis of hyperthermia. Crit Rev Oncol Hematol *43*: 33-56, 2002.
5 Issels RD: Hyperthermia adds to chemotherapy. Eur J Cancer *44*: 2546-2554, 2008.
6 Katschinski DM: On heat and cells and proteins. News Physiol Sci *19*: 11-15, 2004.
7 van der Zee J, Gonzalez Gonzalez D, van Rhoon GC, van Dijk JD, van Putten WL and Hart AA: Comparison of radiotherapy alone with radiotherapy plus hyperthermia in locally advanced pelvic tumours: A prospective, randomised, multicentre trial. Dutch Deep Hyperthermia Group. Lancet *355*: 1119-1125, 2000.
8 Ishizuka M, Abe F, Sano Y, Takahashi K, Inoue K, Nakajima M, Kohda T, Komatsu N, Ogura S and Tanaka T: Novel development of 5-aminolevurinic acid (ALA) in cancer diagnoses and therapy. Int Immunopharmacol *11*: 358-365, 2011.
9 Berlin NI, Neuberger A and Scott JJ: The metabolism of delta - aminolaevulic acid. 1. Normal pathways, studied with the aid of 15N. Biochem J *64*: 80-90, 1956.
10 Berlin NI, Neuberger A and Scott JJ: The metabolism of delta-aminolaevulic acid. 2. Normal pathways, studied with the aid of 14C. Biochem J *64*: 90-100, 1956.
11 Khan AA and Quigley JG: Control of intracellular heme levels: Heme transporters and heme oxygenases. Biochim Biophys Acta *1813*: 668-682, 2011.
12 Frolund S, Marquez OC, Larsen M, Brodin B and Nielsen CU: Delta-aminolevulinic acid is a substrate for the amino acid transporter SLC36A1 (hPAT1). Br J Pharmacol *159*: 1339-1353, 2010.
13 Stummer W, Pichlmeier U, Meinel T, Wiestler OD, Zanella F and Reulen H-J: Fluorescence-guided surgery with 5-aminolevulinic acid for resection of malignant glioma: A randomised controlled multicentre phase III trial. The Lancet Oncology *7*: 392-401, 2006.
14 Inoue K, Fukuhara H, Shimamoto T, Kamada M, Iiyama T, Miyamura M, Kurabayashi A, Furihata M, Tanimura M, Watanabe H and Shuin T: Comparison between intravesical and oral administration of 5-aminolevulinic acid in the clinical benefit of photodynamic diagnosis for nonmuscle invasive bladder cancer. Cancer *118*: 1062-1074, 2012.
15 Grant WE, Hopper C, MacRobert AJ, Speight PM and Bown SG: Photodynamic therapy of oral cancer: Photosensitisation with systemic aminolaevulinic acid. Lancet *342*: 147-148, 1993.
16 Kishi K, Fujiwara Y, Yano M, Inoue M, Miyashiro I, Motoori M, Shingai T, Gotoh K, Takahashi H, Noura S, Yamada T, Ohue M, Ohigashi H and Ishikawa O: Staging laparoscopy using ALA-mediated photodynamic diagnosis improves the detection of peritoneal metastases in advanced gastric cancer. J Surg Oncol *106*: 294-298, 2012.

17 Murayama Y, Ichikawa D, Koizumi N, Komatsu S, Shiozaki A, Kuriu Y, Ikoma H, Kubota T, Nakanishi M, Harada Y, Fujiwara H, Okamoto K, Ochiai T, Kokuba Y, Takamatsu T and Otsuji E: Staging fluorescence laparoscopy for gastric cancer by using 5-aminolevulinic acid. Anticancer Res *32*: 5421-5427, 2012.

18 Talmadge E, Lenz BF, Collins MS, Uithoven KA, Schneider MA, Adams JS, Pearson JW, Agee WJ, Fox RE and Oldham RK: Tumor models to investigate the therapeutic efficiency of immunomodulators. Behring Inst Mitt *74*: 219-29, 1984.

19 Freireich EJ, Gehan EA, Rall DP, Schmidt LH and Skipper HE: Quantitative comparison of toxicity of anticancer agents in mouse, rat, hamster, dog, monkey, and man. Cancer Chemother Rep *50*: 219-244, 1966

20 Ishizuka M, Hagiya Y, Mizokami Y, Honda K, Tabata K, Kamachi T, Takahashi K, Abe F, Tanaka T, Nakajima M, Ogura S and Okura I: Porphyrins in urine after administration of 5-aminolevulinic acid as a potential tumor marker. Photodiagnosis Photodyn Ther *8*: 328-331, 2011.

21 Yamamoto J, Ogura S, Tanaka T, Kitagawa T, Nakano Y, Saito T, Takahashi M, Akiba D and Nishizawa S: Radiosensitizing effect of 5-aminolevulinic acid-induced protoporphyrin IX in glioma cells *in vitro*. Oncol Rep *27*: 1748-1752, 2012.

22 Nonaka M, Yamamoto M, Yoshino S, Umemura S, Sasaki K and Fukushima T: Sonodynamic therapy consisting of focused ultrasound and a photosensitizer causes a selective antitumor effect in a rat intracranial glioma model. Anticancer Res *29*: 943-950, 2009.

23 Yamaguchi F, Asakura T, Takahashi H, Kitamura T and Teramoto A: Low frequency ultrasonication induced antitumor effect in 5-aminolevulinic acid treated malignant glioma. J Cancer Thera *4*: 170-175, 2013.

24 Ohmura T, Fukushima T, Shibaguchi H, Yoshizawa S, Inoue T, Kuroki M, Sasaki K and Umemura S: Sonodynamic therapy with 5-aminolevulinic acid and focused ultrasound for deep-seated intracranial glioma in rat. Anticancer Res *31*: 2527-2533, 2011.

25 Chibazakura T, Toriyabe Y, Takahashi K, Kawakami M, Ogura S, Abe F, Nakajima M and Tanaka T: Enhancement of hyperthermia-induced tumor cell death by 5-aminolevulinic acid. *In*: The 11th International Congress of Hyperthemic Oncology (Proceeding). Kyoto, 2012.

26 Morandi B, Mortara L, Chiossone L, Accolla RS, Mingari MC, Moretta L, Moretta A and Ferlazzo G: Dendritic cell editing by activated natural killer cells results in a more protective cancer-specific immune response. PLoS One *7*: e39170, 2012.

27 Hinuma S, Naruo K, Ootsu K, Houkan T, Shiho O and Tsukamoto K: Suppression of pulmonary tumour metastasis in mice by recombinant human interleukin-2: Role of asialo GM1-positive cells. Immunology *60*: 173-179, 1987.

28 Skivka LM, Gorobets OB, Kutsenok VV, Lozinsky MO, Borisevich AN, Fedorchuk AG, Kholin VV and Gamaleya NF: 5-Aminolevulinic acid mediated photodynamic therapy of Lewis lung carcinoma: A role of tumor infiltration with different cells of immune system. Exp Oncol *26*: 312-315, 2004.

29 Moretta A, Marcenaro E, Parolini S, Ferlazzo G and Moretta L: NK cells at the interface between innate and adaptive immunity. Cell Death Differ *15*: 226-233, 2008.

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